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Foot-and-Mouth Disease Virus Assembly: Processing of Recombinant Capsid Precursor by Exogenous Protease Induces Self-Assembly of Pentamers In Vitro in a Myristoylation-Dependent Manner[▽]

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The assembly of foot-and-mouth disease virus (FMDV) particles is poorly understood. In addition, there are important differences in the antigenic and receptor binding properties of virus assembly and dissociation intermediates, and these also remain unexplained. We have established an experimental model in which the antigenicity, receptor binding characteristics, and in vitro assembly of capsid precursor can be studied entirely from purified components. Recombinant capsid precursor protein (P1 region) was expressed in *Escherichia coli* as myristoylated or unmyristoylated protein. The protein sedimented in sucrose gradients at 5S and reacted with monoclonal antibodies which recognize conformational or linear antigen determinants on the virion surface. In addition, it bound the integrin $\alpha_v\beta_6$, a cellular receptor for FMDV, indicating that unprocessed recombinant capsid precursor is both structurally and antigenically similar to native virus capsid. These characteristics were not dependent on the presence of 2A at the C terminus but were altered by N-terminal myristoylation and in mutant precursors which lacked VP4. Proteolytic processing of myristoylated precursor by recombinant FMDV $3C^{pro}$ in vitro induced a shift in sedimentation from 5S to 12S, indicating assembly into pentameric capsid subunits. Nonmyristoylated precursor still assembled into higher-order structures after processing with $3C^{pro}$, but these particles sedimented in sucrose gradients at approximately 17S. In contrast, mutant precursors lacking VP4 were antigenically distinct, were unable to form pentamers, and had reduced capacity for binding integrin receptor. These studies demonstrate the utility of recombinant capsid precursor protein for investigating the initial stages of assembly of FMDV and other picornaviruses.

Foot-and-mouth disease virus (FMDV) is the etiological agent of a highly infectious disease of cattle and other cloven-hoofed animals. It is of economic importance, because the presence of the disease results in severe restrictions of international trade. In many parts of the developing world, the disease is endemic, and it continues to pose a serious threat to livestock industries globally, as exemplified by recent major outbreaks in the United Kingdom, Argentina, and Uruguay.

FMDV is a small, nonenveloped, positive-strand RNA virus belonging to the genus *Aphthovirus* within the family *Picornaviridae*. Other members of the family include poliovirus (PV; genus *Enterovirus*) and hepatitis A virus (genus *Hepatovirus*). The mature picornavirus comprises 60 copies each of four structural proteins, termed VP1, VP2, VP3, and VP4, which encapsidate a single, positive-sense RNA genome. The proteins form a pseudo $T=3$ icosahedral capsid with VP1 located close to the fivefold axes of symmetry and VP2 and VP3 alternating around the threefold axes (24). VP4, which is myristoylated at the N terminus, is an internal component of the capsid (13, 48).

Although the morphogenesis of picornaviral particles is not completely understood, it is known that capsid assembly proceeds from a monomeric precursor, via a pentameric intermediate, to the intact icosahedral capsid. For enteroviruses such as PV, the capsid precursor is called P1 and contains the four structural proteins VP1 to VP4 as an unprocessed polyprotein. However, in some other picornavirus genera, including the aphthoviruses, the capsid precursor has an additional C-terminal extension and is known as P1-2A (46). In hepatitis A virus, the 2A component is involved in the particle assembly mechanism, although it is absent from the mature virion (15, 42, 51). However, the role of 2A in the assembly of FMDV is unclear.

Processing by the viral protease $3C^{pro}$ (aphthoviruses) or 3CD (enteroviruses) cleaves the precursor polyprotein into VP0, VP1, and VP3. These three components remain associated in a protein complex which now acts as a monomer for the self-assembly of five monomers into the pentameric capsid subunit. The assembly of higher-order structures from monomeric subunits has been shown for several picornaviruses to be dependent on N-terminal myristoylation of the precursor (1, 36, 37, 43). The myristoyl moieties in the mature PV are clustered near the fivefold vertices on the capsid interior and participate in stabilizing interactions between the five protomer subunits that form the pentamer (13, 27). In infected cells, incompletely myristoylated pentamers are thought to be excluded from virus capsids (36, 37, 43). However, the ability of

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assembled particles to tolerate a degree of incorporation of unmyristoylated material has also been reported (4).

The further assembly of 12 pentamers and a molecule of genomic RNA generates a provirion (2, 33), which subsequently undergoes maturation cleavage to convert VP0 into VP2 and VP4 to produce the mature infectious virion (5, 26, 32, 52). A number of picornaviruses, including FMDV, also have the ability to form empty capsids which have the same antigenicity as virions but do not contain RNA, do not usually contain cleaved VP0, and are less stable than virions (5, 10, 17, 44, 49, 53, 54, 58).

The assembly intermediates detected in infected cells can be distinguished by their sedimentation as the 5S capsid precursor (monomer), the 12S (FMDV) or 14S (PV) pentamer, the 75S (FMDV) or 80S (PV) empty capsid, and the 140S (FMDV) or 160S (PV) virion.

For many picornaviruses, receptor binding occurs within pits or depressions on the virion surface. In contrast, the receptor binding feature of FMDV is a long and flexible sequence (the VP1 G-H loop) that protrudes from the otherwise smooth surface of the particle (24). The tripeptide Arg-Gly-Asp is found at the distal end of this loop and is the signature recognition motif for the integrin receptor $\alpha_v\beta_6$, used by the virus for cell attachment (29). The VP1 G-H loop is also a major antigenic feature of the virus, and synthetic peptides representing this sequence can elicit neutralizing and protective antibody responses (23, 56). Receptor binding and antigenic characteristics of the G-H loop are influenced by subtle differences in its association with the remainder of the capsid in ways that are poorly understood. For example, mutations that confer resistance to anti-G-H loop neutralizing monoclonal antibodies (MAbs) have been observed in distal regions of the capsid and appear to induce changes in the orientation of the loop with respect to the capsid surface (47).

Receptor binding induces internalization of FMDV into early endosomes (7) where the pH is sufficiently low to induce acid-mediated dissociation of the particle (16, 22). This results in the separation of pentameric subunits, consisting of five copies each of VP1 to VP3, with release of the RNA and of the internally located VP4. Pentamers formed by dissociation of virus therefore differ from those of the assembly pathway in that they lack the VP4 component. Pentamers and "artificial" empty particles (on the dissociation pathway from mature virions and lacking VP4) are also poor immunogens and do not bind efficiently to cells relative to virions and "natural" empty particles (on the assembly pathway and containing VP4) (11, 12, 57; D. V. Sangar, unpublished data). These observations suggest that VP4 plays an important role in determining the antigenic status of the capsid and influences receptor binding by the G-H loop.

Earlier studies demonstrated the production of empty capsids of several picornaviruses following translation of the viral RNA in rabbit reticulocyte lysates (25, 30, 45) or expression of capsid precursor in bacterial (34), yeast (55), insect (14) and mammalian (1, 3) cells. In these studies, capsid precursors were coexpressed with the viral protease in order for capsid assembly to occur within the expression system. In addition, the formation of empty capsids has been shown by the self-assembly of native pentamers purified from infected cells (50, 54). To study early events in the assembly process in greater detail, we established an experimental model in which the in vitro processing and assembly of FMDV capsid intermediates can be studied entirely from purified recombinant components.

Here we report the antigenic characterization of purified recombinant capsid precursor proteins and the cell-free assembly of 12S pentameric capsid subunits that are structurally similar to those of native pentamers observed in infected cells. Assembly of authentic 12S pentamers was dependent on proteolytic cleavage by $3C^{pro}$ and required N-terminal myristoylation of the precursor but was independent of 2A. Abnormal assembly occurred in the absence of N-terminal myristoylation, and no assembly occurred if VP4 was not present. The presence or absence of VP4 also affected both the antigenic and receptor binding properties of the precursor protein.

MATERIALS AND METHODS

DNA constructs. A subgenomic cDNA clone encoding the structural proteins of FMDV serotype CS8-c1 (accession no. AJ133357) was kindly provided by E. Domingo. Constructs encoding either P1 (nucleotides 1642 to 3832) or P1-2A (nucleotides 1642 to 3881) were designed from data reported by Toja et al. (60). Thirty-two rare codons (significantly, ATA [isoleucine], CTC and CTA [leucine], and AGG and AGA [arginine]) that did not match the synonymous codon usage of *E. coli* were modified by overlap PCR mutagenesis, and products were sequenced and cloned into expression vector pET23a+ (Novagen, Merck Biosciences). The plasmid pET28a3C, encoding the soluble $3C^{pro}$ (C95K/C142L) mutant, was kindly provided by S. Curry. Plasmid pET28bNMT, encoding human *N*-myristoyl transferase, was a kind gift from R. Clegg.

MAbs. A number of well-characterized MAbs directed against different epitopes on FMDV CS8-C1 particles (38–41) were used in this study. Neutralizing MAb SD6 recognizes the βG-βH hypervariable loop of VP1, while MAbs 5C4 and 2E5 recognize conformation-specific discontinuous epitopes at the pseudo-threefold axis. Additional MAbs recognizing the B-C loop of VP2 (6F2) and the B-B "knob" of VP3 (6C2) were used to identify individual structural proteins. The antibodies were harvested as culture supernatants from hybridoma cell lines (kindly provided by E. Domingo).

Expression and purification of recombinant protein. Competent *E. coli* BL21(DE3)(pLysS STAR) cells were transformed with plasmid PET23aP1 or PET23aP1-2A or derivatives thereof (see below). Cultures were grown at 37°C in Luria-Bertani (LB) broth with 100 μg/ml ampicillin until an optical density at 600 nm of 0.6 was reached and induced with 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 4 h at 37°C. For the production of myristoylated proteins, bacteria were cotransformed with pET28bNMT (kanamycin resistant) and supplemented with 5 μg/ml myristic acid. Unmyristoylated proteins were produced by expression in the absence of pET28bNMT or from constructs in which the myristoylation signal had been mutated (G2A). The cells were harvested by centrifugation and disrupted using a French press. Recombinant proteins were engineered with a C-terminal six-histidine tag and were recovered from supernatants by immobilized nickel-ion affinity chromatography. Recombinant modified $3C^{pro}$ was similarly expressed and purified from plasmid pET28a3C.

Cell-free translation. Capsid precursor proteins were translated in vitro from plasmid DNA in a rabbit reticulocyte cell-free transcription/translation system (Promega) according to the manufacturer's instructions. Radiolabeled proteins were made by including [^{35}S]methionine or [^{3}H]myristic acid (Amersham Biosciences) as appropriate. Translation of human immunodeficiency virus (HIV) nef was used as a positive control for incorporation of labeled myristic acid (6) and used plasmid constructs kindly provided by M. Bentham and M. Harris.

In vitro proteolysis. In vitro $3C^{pro}$ reactions were performed in cleavage buffer (100 mM HEPES [pH 7.5], 500 mM NaCl, 1 mM EDTA, 1 mM β-mercaptoethanol) at 30°C for up to 16 h. The reaction mixtures included 100 to 500 μg of capsid precursor protein and $3C^{pro}$ at a molar ratio of 1:2.

ELISA. For enzyme-linked immunosorbent assays (ELISAs), plastic 96-well plates were coated with sample proteins (50 μg/ml) in coating buffer (100 mM HEPES [pH 7.5], 500 mM NaCl) for 16 h at 4°C. The plates were washed with binding buffer (phosphate-buffered saline [PBS], 0.1% Tween-20, 0.2% bovine serum albumin) and blocked with blocking buffer (PBS, 2% bovine serum albumin) at room temperature for 2 h. After three washes with binding buffer, the proteins were reacted with MAbs in binding buffer for 1 h at room temperature. The plates were again washed with binding buffer, and bound antibody was

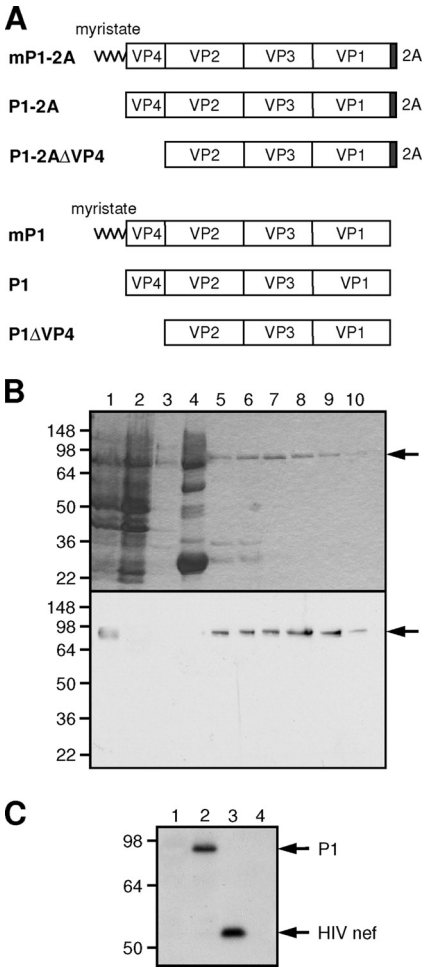

FIG. 1. Capsid precursor protein production. (A) Depiction of the six different forms of precursor protein produced, covering all combinations of myristate and/or VP4 at the N terminus and 2A at the C terminus. (B) Purification of recombinant precursor protein (unmyristoylated P1-2A) shown by SDS-PAGE and Coomassie stain (top) or immunoblot (bottom). Lane 1, bacterial lysate; lanes 2 to 4, column flowthrough and wash; lanes 5 to 10, column elution. Molecular size markers (kDa) are on the left. Arrows indicate the position of precursor protein. Equivalent results were obtained with each form of precursor. No expression was detected in uninduced cultures. (C) Incorporation of radiolabeled myristic acid during cell-free translation, shown by SDS-PAGE and autoradiography. Lane 1, capsid precursor containing a mutated defective myristoylation signal (G2A); lane 2, capsid precursor containing wild-type myristoylation signal; lane 3, HIV Nef-GST fusion protein; lane 4, HIV Nef-GST containing a mutated defective myristoylation signal (G2A). Molecular size markers (kDa) are on the left.

detected with a goat anti-mouse horseradish peroxidase-conjugated polyclonal antiserum (Sigma). Complexed protein was then detected using orthophenyl diamine-hydrogen peroxide substrate (DakoCytomation) as per the manufacturer's instructions. The reaction was quenched with 100 mM H_2SO_4, and the plate was read by absorbance at 490 nm. In receptor binding studies, the plates were coated with purified recombinant integrin $\alpha_v\beta_6$ (50 μg/ml) to capture recombinant precursor protein, which was then detected as described above. Integrin $\alpha_v\beta_6$ was generously provided by N. Abrescia. Background ELISA signal was determined by using mock samples, mock receptor coating, or mock primary antibody treatment, all of which consistently gave very low signals. This background value was subtracted from all the ELISA data shown.

SDS-PAGE and immunoblot analysis. Samples were resolved by 12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and either stained by Coomassie blue or transferred to polyvinylidene difluoride membranes (Millipore) using a semidry blotter (Bio-Rad). After blocking in 5% (wt/vol) nonfat milk, membranes were incubated with primary antibody (SD6, 6F2, 6C2, or α-HIS) diluted 1:1,000 in PBS with 2% (wt/vol) nonfat milk solution. Bound immunoglobulin was detected with HRP-conjugated goat anti-mouse immunoglobulin G (Sigma) diluted 1:5,000 in PBS, and protein was visualized by standard chemiluminescence techniques.

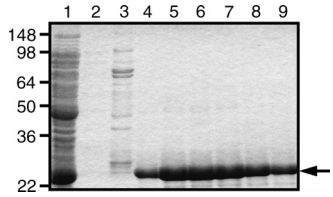

FIG. 2. Protease $3C^{pro}$ purification shown by SDS-PAGE and Coomassie stain. Lane 1, bacterial lysate; lanes 2 to 3, column wash; lanes 4 to 9, column elution. Molecular size markers (kDa) are on the left. The arrow indicates the position of $3C^{pro}$. No expression was detected in uninduced cultures.

Sucrose density centrifugation. Continuous gradients of 5 to 30% sucrose in 100 mM HEPES (pH 7.5), 500 mM NaCl were loaded with samples and centrifuged at 150,000 × *g* for 18 h at 4°C in a Sorvall AH-650 rotor. Fractions were collected from the bottom of the gradient, and viral proteins were detected by ELISA with MAb 5C4.

Autoradiography. SDS-PAGE gels were placed in 40% (vol/vol) methanol, 10% (vol/vol) glacial acetic acid for 20 min and fluorography reagent (Amplify; GE Healthcare) for 30 min. The gels were dried on 3MM paper (Whatman) and exposed to film at −80°C for 12 to 72 h.

RESULTS

Expression and purification of the capsid precursor protein and 3C protease ($3C^{pro}$) of FMDV. The capsid precursor protein constructs used in this study are depicted in Fig. 1A. Each construct was extended at the C terminus with a six-histidine tract to facilitate purification using a nickel ion-charged resin following expression in *E. coli*. Purified precursor protein migrated in SDS-PAGE as expected with an apparent molecular mass of 88 kDa (Fig. 1B) and was recognized in Western blots by the anti-FMDV type C reactive nonconformational MAb SD6 (Fig. 1B). The natural precursor protein is myristoylated at the N terminus. The same modification of recombinant proteins in *E. coli* is achieved by the coexpression of *N*-myristoyl transferase (21), a system used routinely in our laboratory for the production of myristoylated proteins (6, 18, 19). The myristoylation of capsid precursor was also tested by the incorporation of radiolabeled myristic acid into proteins expressed in cell-free translation reactions (Fig. 1C).

The viral protease, $3C^{pro}$, was also constructed with a His tag for purification purposes and expressed as for P1. Initial experiments showed that natural $3C^{pro}$ protein could be highly expressed and purified, but it rapidly aggregated and could not be used for in vitro processing experiments. This problem was overcome by the expression of a modified $3C^{pro}$ from serotype A FMDV (Fig. 2) which contained two substitutions (C95K and C142L) which greatly improved solubility while retaining activity of the enzyme (8, 9).

Capsid precursor proteins are recognized by conformational and nonconformational antibodies and by integrin $\alpha_v\beta_6$. Each of the precursor proteins derived from the constructs depicted in Fig. 1A was tested by ELISA for recognition

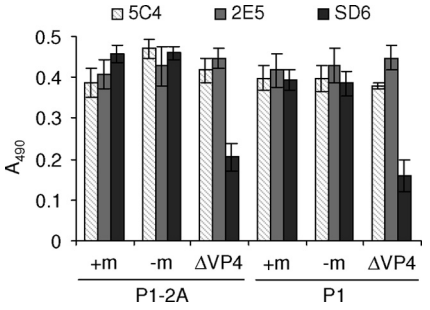

FIG. 3. Capsid precursor proteins are recognized by MAbs against mature virus. ELISA showed the reactivity of the six forms of capsid precursor (Fig. 1A) with antibodies 5C4 and 2E5 (discontinuous epitopes) and SD6 (G-H loop). Results for myristoylated capsid precursor (+m), unmyristoylated capsid precursor (−m), and capsid precursor omitting VP4 (ΔVP4) are shown. Error bars represent standard deviations derived from quadruplicate samples.

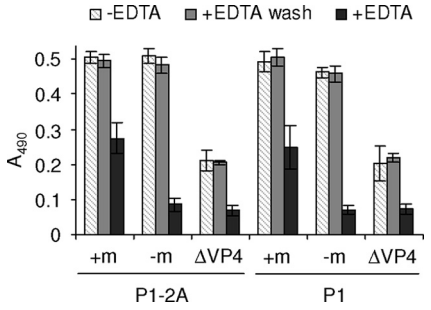

FIG. 4. Binding of capsid precursor to the virus receptor integrin $\alpha_v\beta_6$ is dependent on precursor composition and is inhibited by EDTA. Capture ELISA showed binding of the six forms of capsid precursor (Fig. 1A) to integrin-coated plates in the presence and absence of EDTA (+EDTA and −EDTA) or binding in the absence/washing in the presence of EDTA (+EDTA wash). Results for myristoylated capsid precursor (+m), unmyristoylated capsid precursor (−m), and capsid precursor omitting VP4 (ΔVP4) are shown. Error bars represent standard deviations derived from quadruplicate samples.

by three MAbs raised against FMDV serotype C particles. MAb SD6 recognizes the VP1 G-H loop sequence (antigenic site A) in a non-conformation-dependent manner, while MAbs 5C4 and 2E5 bind discontinuous epitopes located at the threefold axis of the virus (antigenic site D) (39, 40). Proteins were recognized equally by each of the antibodies irrespective of their myristoylation at the N terminus or the presence of 2A at the C terminus (Fig. 3). These results confirm earlier observations that the uncleaved precursor protein robustly adopts a fold similar to that of the fully processed proteins within the virus capsid (59). In contrast, recognition by MAb SD6 of proteins that lacked the VP4 component was significantly reduced, although binding of the two conformational antibodies was unaltered (Fig. 3).

The ability of the recombinant proteins to bind the FMDV receptor integrin $\alpha_v\beta_6$ was also assessed in a modified ELISA in which integrin was bound to the plate to capture the proteins. Binding was demonstrated by reaction with MAb 5C4. The binding of ligands to integrins such as $\alpha_v\beta_6$ is dependent on Ca^{2+} and can be inhibited in the presence of EDTA. However, it was recently shown that once bound, the high-affinity association of the VP1 G-H loop sequence of FMDV is not reversed in the presence of EDTA (20). Consequently, the binding of the recombinant proteins to integrin was determined in the presence or absence of EDTA. The resistance of capsid precursor-integrin complexes to reversal was also assessed by washing with buffer containing EDTA. The results (Fig. 4) show that in the absence of EDTA, precursor proteins bound immobilized integrin equally well irrespective of their myristoylation status and independent of the presence of 2A. Binding of nonmyristoylated precursors was reduced by approximately 80% in the presence of EDTA. By contrast, receptor binding by the myristoylated versions of these proteins was reduced by only approximately 50% in the presence of EDTA. Interestingly, proteins from which the VP4 moiety had been deleted bound to the integrin much less efficiently than full-length proteins even in the absence of EDTA. Once bound to receptor, all of the proteins were resistant to removal by washing with EDTA-containing buffer, consistent with previously reported findings (20).

Soluble recombinant precursor proteins sediment as monomeric species in sucrose density gradients. Although each of the recombinant proteins produced in this study could be purified from bacterial lysates as soluble material by affinity chromatography, we wished to determine their monomeric/oligomeric status. This was assessed by centrifugation through sucrose density gradients using a series of marker proteins separated in parallel gradients to estimate sedimentation coefficients. The results in Fig. 5 clearly show that each of the proteins sediments as a monomeric species at approximately 5S irrespective of its myristoylation status and independent of

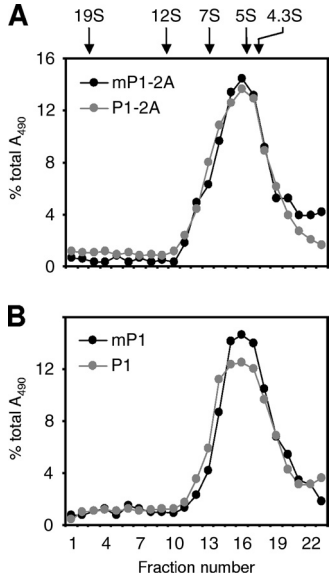

FIG. 5. Sedimentation of capsid precursor proteins. ELISA of density gradient fractions was done after ultracentrifugation of capsid precursors containing 2A (P1-2A) (A) or capsid precursors omitting 2A (P1) (B). Capsid precursor nomenclature is as shown in Fig. 1A. Gradients were fractionated from the bottom. The positions in gradients of marker proteins with known sedimentation values are indicated at the top of panel A.

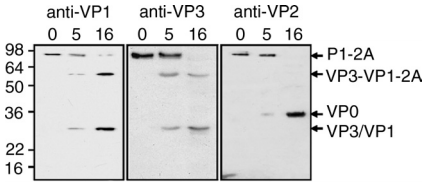

FIG. 6. Proteolytic processing of capsid precursor by viral protease $3C^{pro}$. SDS-PAGE and immunoblotting show the time course of capsid precursor cleavage and identification of products. Time points are in hours. Molecular size markers (kDa) are on the left.

the presence of 2A. No evidence of assembly into higher-order oligomeric structures was seen under these conditions.

In vitro processing of recombinant capsid precursor by viral protease $3C^{pro}$. Purified capsid precursor proteins were incubated with purified recombinant FMDV $3C^{pro}$, and aliquots were taken at intervals for analysis by SDS-PAGE and immunoblotting. The individual processed capsid proteins were identified using antibodies specific for capsid proteins VP1, VP2, and VP3. Results of an experiment with precursor protein containing 2A are shown in Fig. 6. Similar results were obtained with other recombinant proteins and were unaffected by the presence of myristate or 2A (data not shown). There was a progressive cleavage of the precursor protein into the expected products VP0 (recognized by the anti-VP2 antibody), VP3, and VP1. By 16 h, the cleavage at the VP0/VP3 junction had gone to completion, whereas there was still a small (variable) amount of uncleaved VP3-VP1 precursor. From these observations we conclude that the order of cleavage is VP0/VP3 (relatively rapid) followed by VP3/VP1 (slow). Further cleavage after incubation beyond 16 h was not observed.

Assembly of processed capsid precursor into pentameric capsid subunits. The consequences of proteolytic processing on the self-assembly of structural precursor proteins were investigated by sedimentation through sucrose density gradients and analysis of gradient fractions in ELISA using MAb 5C4 for detection. After cleavage of myristoylated protein (mP1-2A), a new peak with a sedimentation coefficient of approximately 12S was detected (Fig. 7A), suggesting that the processed precursors could assemble into pentameric subunits similar to those seen during natural infection. The proportion of processed precursor protein that was assembled into the 12S form varied from 10 to 50% in individual experiments. No material could be recovered from the bottoms of the centrifuge tubes, suggesting that further assembly of the pentameric subunits into higher-order structures such as empty capsids did not occur under the conditions used in these experiments. This is consistent with previous studies of self-assembly of PV empty capsids, where a critical threshold concentration of pentamers was required before capsid formation could occur (54). In the present study, the pentamer concentration was maintained below this critical threshold, thus permitting specific analysis of the protomer-to-pentamer assembly step.

Processing of precursor protein lacking N-terminal myristoylation (P1-2A) also facilitated assembly into specific higher-order structures, but in contrast to the 12S particle formed from myristoylated precursor, the unmyristoylated material assembled into a particle with a sedimentation coefficient of approximately 17S (Fig. 7B). The 17S material was clearly still

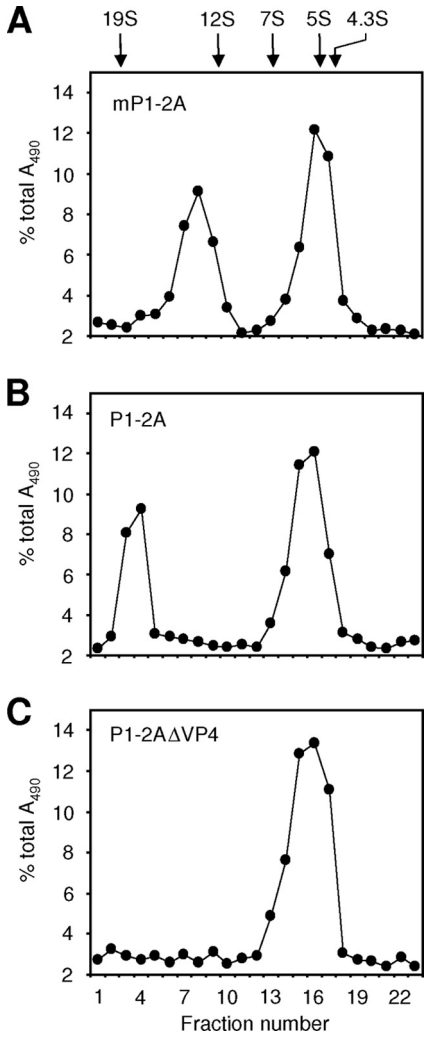

FIG. 7. Assembly of processed capsid precursor into pentameric capsid subunits. ELISA of density gradient fractions was done after ultracentrifugation of cleavage reaction products containing myristoylated capsid precursor (mP1-2A) (A), unmyristoylated capsid precursor (P1-2A) (B), and capsid precursor omitting VP4 (P1-2A ΔVP4) (C). Gradients were fractionated from the bottom. The positions in gradients of marker proteins with known sedimentation values are indicated at the top of panel A.

reactive with the conformation-dependent antibodies used for their detection. Again, no evidence for the assembly of empty capsids was detected by analysis of material at the bottom of centrifuge tubes.

Parallel experiments were carried out with myristoylated and unmyristoylated forms of precursor which lacked 2A (P1), and identical results were obtained as described above for precursor P1-2A. This strongly suggests that the presence of 2A is not required for the assembly of FMDV pentamers.

In contrast to what was observed with full-length precursor proteins, no assembly was detected following sucrose gradient analysis of processed proteins which lacked the VP4 component (P1-2AΔVP4) (Fig. 7C). Western blot analysis of the samples used in assembly experiments confirmed that appropriate $3C^{pro}$-mediated processing had taken place, consistent

with data shown in Fig. 6. The confirmation of processing of P1-2AΔVP4 ruled out the possibility that loss of substrate function had prevented the assembly of this material.

DISCUSSION

Proteolytic cleavage and subsequent assembly of picornavirus structural proteins are incompletely understood, and we report here a system in which these processes can be studied in vitro for FMDV using purified recombinant proteins. This has allowed us to investigate the inherent properties of both the natural and mutated forms of the capsid precursor protein with respect to antigenicity, receptor binding, and self-assembly into higher-order complexes. The capsid precursor proteins of most picornaviruses (except, for example, hepato- and parechoviruses) are modified by the covalent addition of a myristate group at the N terminus, and there is evidence that this plays a role in their assembly into pentameric viral subunits (1, 36, 37, 43). The C termini of the capsid precursor proteins of different genera of the picornaviruses differ with respect to the presence or absence of the nonstructural protein, 2A. In the enteroviruses, a primary cleavage that separates the structural protein region from the remainder of the polyprotein occurs at the C terminus of the VP1 domain, and no nonstructural proteins are included in the capsid precursor. However, in other genera, e.g., cardioviruses, aphthoviruses, and hepatoviruses, a primary cleavage event occurs at the C terminus of 2A so that the capsid precursor protein comprises P1-2A (46). The role of 2A, if any, in the subsequent processing and assembly of the structural proteins of cardio- and aphthoviruses is unclear, but for hepatoviruses, it plays an important role in the assembly process, although it is cleaved away from VP1 in the mature virion (15, 42, 51). Finally, the cleavage of VP0 into VP2 and VP4 occurs at the final stage of virus particle maturation and has important effects on particle stability, priming the particle in a metastable state in preparation for conformational changes involved in the cell entry process required to establish a new infection (17). As stated in the introduction, there is evidence that the presence or absence of VP4 has important effects on the antigenicity and receptor binding properties of virus and empty particles (57), but the structural basis for these differences is not understood.

To investigate these issues we produced (i) myristoylated or unmyristoylated forms of the P1 polyprotein of FMDV, serotype C, in *E. coli*, (ii) P1 with or without the C-terminal extension of 2A (P1-2A), and (iii) P1 from which the VP4 region had been deleted (P1ΔVP4). Each protein had a C-terminal extension of six histidine residues to facilitate purification by nickel ion chromatography. In the crystal structure of FMDV (24), the C terminus of VP1 is located on the external surface of the particle, and we reasoned that the presence of the His tag at this position would cause minimal structural perturbation.

Each of the proteins could be purified from *E. coli* lysates as soluble material which sedimented in sucrose gradients at approximately 5S, the expected sedimentation coefficient of monomeric capsid precursor, with no evidence of assembly into higher-order structures. Each of the constructs was recognized equally by two MAbs that bind to a conformation-dependent discontinuous antigenic site (site D) located at the pseudo-threefold axis of symmetry of the virus particle. This indicates that the native folding characteristics of the structural proteins are largely defined within the uncleaved precursor protein and are not dependent on subsequent proteolytic processing, in agreement with earlier studies (59).

MAb SD6, which recognizes a linear epitope located on the VP1 G-H loop, also bound well to unprocessed P1 proteins but significantly less well to P1 proteins from which the N-terminal VP4 domain had been omitted. This is counterintuitive, as the VP1 G-H loop, which bears the cell receptor binding motif, is flexibly attached at the surface of the virus particle, suggesting that it might function as an independent unit. However, earlier studies had shown that mutations effecting the orientation of the VP1 G-H loop on the virus surface can influence recognition by MAbs directed against the feature (47). In addition, pentameric subunits derived by acid- or heat-induced dissociation of virus particles still carry the VP1 G-H loop sequence but lack the ability to block virus binding to cells (Sangar, unpublished), presumably due to their reduced ability to bind receptor. As with P1-ΔVP4, the pentameric subunits derived by dissociation of virus particles lack VP4. Taken together, these observations suggest that the presence of VP4 may subtly alter the structure of the VP1 G-H loop to affect its biological properties.

Each of the unprocessed precursor proteins was able to bind to purified integrin $\alpha_v\beta_6$ in a capture ELISA. Integrin binding to a majority of ligands is regulated by the presence of divalent cations (28, 31, 35), and in agreement with the known requirements for Ca^{2+}, EDTA significantly reduced binding by the precursor proteins. However, the reduction of binding in the presence of EDTA was less pronounced for myristoylated versions of the proteins, suggesting that this posttranslational modification may also indirectly alter the structure of the VP1 G-H loop to increase the affinity of receptor binding even in the absence of Ca^{2+}.

It is interesting that attachment to receptor was only initially dependent on the presence of cations and that, after binding, washing with EDTA-containing buffer did not reverse the receptor ligand interaction. A similar observation had been made with synthetic peptides representing the FMDV VP1 G-H loop (20). The role of divalent cations in ligand binding to integrins is to mediate extensive structural rearrangements within extracellular domains of the receptor (so-called "switchblade-like" movement). These alterations drive the integrin from a low-affinity to a high-affinity conformation. However, these results demonstrate that FMDV capsid precursor, like synthetic peptides corresponding to the G-H loop of VP1 (20), is capable of maintaining integrin $\alpha_v\beta_6$ in a high-affinity state which either no longer needs the presence of divalent cations to maintain binding of the ligand or complexes the cations such that they can no longer be removed by EDTA.

In vitro processing of the purified precursor proteins by wild-type $3C^{pro}$ was not possible, since although the protein was expressed at a high level and could be readily purified using Ni^{2+} chromatography, it rapidly aggregated and became insoluble. This problem had hindered attempts to solve the crystal structure of the enzyme but was overcome by the introduction of mutations at the surface of the protein which allowed it to maintain solubility and proteolytic activity (8, 9). This modified protease was able to process capsid precursor proteins to near completion, albeit at a low rate, presumably

due to reduced activity compared to the wild-type enzyme (capsid precursor polyprotein would normally be processed to completion by native protease in infected cells). There was a clear hierarchy of cleavage in vitro, with the VP0-VP3 and VP1-2A sites being processed more rapidly that the VP3-VP1 site.

Proteolytic processing of the precursor proteins facilitated their assembly into higher-order structures. Sucrose density gradient analyses of processed myristoylated P1 or P1-2A showed that up to 50% of material now sedimented at approximately 12S, the sedimentation coefficient of native pentameric viral subunits. Unmyristoylated processed P1 or P1-2A also assembled into specific larger structures, but these sedimented at approximately 17S. We cannot say whether these particles are pentamers but with a different structure which causes them to sediment faster than 12S particles or whether they contain additional monomers. The myristate moieties are located on the internal surface of picornavirus particles at the axis of fivefold symmetry, and it is tempting to speculate that their presence induces conformational changes which cause the pentamer to "open" into an umbrella shape and impede its sedimentation through sucrose gradients, reducing it from 17S to 12S.

In no case was material found to be sedimenting faster than 12S or 17S, either within the gradients or pelleted at the bottoms of the tubes, indicating that assembly of 80S empty particles did not occur. This is consistent with the concentration of pentameric subunits remaining below that required for capsid self-assembly (54).

In conclusion, we report here the development of a system that facilitates study of the processing and assembly of the capsid precursor protein of FMDV using purified components and under defined conditions. Investigations involving mutated forms of the proteins have illustrated some of the structural requirements necessary for assembly and the subtle alterations they can induce in the biological properties of the proteins. Most striking is that the presence or absence of VP4 appears to act as a switch, modulating the antigenic, receptor binding, and assembly characteristics of FMDV capsid subunits.

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